

## Sensitivity, Specificity, and Clinical Value of Human Papillomavirus (HPV) E6/E7 mRNA Assay as a Triage Test for Cervical Cytology and HPV DNA Test<sup>▽</sup>

Maria Benevolo,<sup>1</sup> Amina Vocaturo,<sup>1</sup> Donatella Caraceni,<sup>2</sup> Deborah French,<sup>3</sup> Sandra Rosini,<sup>4</sup> Roberta Zappacosta,<sup>4</sup> Irene Terrenato,<sup>5</sup> Lucia Ciccocioppo,<sup>2</sup> Antonio Frega,<sup>6</sup> and Paolo Giorgi Rossi<sup>7\*</sup>

*Pathology Department, Regina Elena Cancer Institute, Rome, Italy<sup>1</sup>; Cytopathology Unit, F. Renzetti Hospital, Lanciano, Chieti, Italy<sup>2</sup>; Clinical and Molecular Medicine Department, 2nd Medicine Faculty of La Sapienza University, Sant'Andrea Hospital, Rome, Italy<sup>3</sup>; Oncology and Neurosciences Department, Cytopathology Section, G. D'Annunzio University, Chieti, Pescara, Italy<sup>4</sup>; Epidemiology Department, Regina Elena Cancer Institute, Rome, Italy<sup>5</sup>; Gynecology Department, 2nd Medicine Faculty of La Sapienza University, Sant'Andrea Hospital, Rome, Italy<sup>6</sup>; and Agency for Public Health Lazio Sanità, Rome, Italy<sup>7</sup>*

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There is evidence that testing for human papillomavirus (HPV) E6/E7 mRNA is more specific than testing for HPV DNA. A retrospective study was carried out to evaluate the performance of the PreTect HPV-Proofer E6/E7 mRNA assay (Norchip) as a triage test for cytology and HPV DNA testing. This study analyzed 1,201 women, 688 of whom had a colposcopy follow-up and 195 of whom had histology-confirmed high-grade intraepithelial neoplasia or worse (CIN2+). The proportion of positive results and the sensitivity and specificity for CIN2+ were determined for HPV mRNA in comparison to HPV DNA and cytology. All data were adjusted for follow-up completeness. Stratified by cytological grades, the HPV mRNA sensitivity was 83% (95% confidence interval [CI] = 63 to 94%) in ASC-US (atypical squamous cells of undetermined significance), 62% (95% CI = 47 to 75%) in L-SIL (low-grade squamous intraepithelial lesion), and 67% (95% CI = 57 to 76%) in H-SIL (high-grade squamous intraepithelial lesion). The corresponding figures were 99, 91, and 96%, respectively, for HPV DNA. The specificities were 82, 76, and 45%, respectively, for HPV mRNA and 29, 13, and 4%, respectively, for HPV DNA. Used as a triage test for ASC-US and L-SIL, mRNA reduced colposcopies by 79% (95% CI = 74 to 83%) and 69% (95% CI = 65 to 74%), respectively, while HPV DNA reduced colposcopies by 38% (95% CI = 32 to 44%) and by 15% (95% CI = 12 to 19%), respectively. As a HPV DNA positivity triage test, mRNA reduced colposcopies by 63% (95% CI = 60 to 66%), having 68% sensitivity (95% CI = 61 to 75%), whereas cytology at the ASC-US+ threshold reduced colposcopies by 23% (95% CI = 20 to 26%), showing 92% sensitivity (95% CI = 87 to 95%). In conclusion, PreTect HPV-Proofer mRNA can serve as a better triage test than HPV DNA to reduce colposcopy referral in both ASC-US and L-SIL. It is also more efficient than cytology for the triage of HPV DNA-positive women. Nevertheless, its low sensitivity demands a strict follow-up of HPV DNA positive-mRNA negative cases.

Due to the wide use of Papanicolaou (Pap) test and implementation of screening programs in industrialized countries, cervical cancer incidence is kept under control, and the prevalence of clinically relevant lesions in the population is currently very low (12, 21). The introduction of the human papillomavirus (HPV) DNA test has further improved the sensitivity of cervical screening (10, 11, 16). However, neoplastic transformation is a rare complication of HPV infection which, in the majority of cases, is a transient event (13). Consequently, the high-risk (HR) HPV test has a low specificity (10, 11) in detecting high-grade cervical intraepithelial neoplasia (CIN2 or CIN3). Randomized controlled trials have shown the effectiveness of screening with the HR-HPV test in reducing cervical cancer mortality and incidence (16, 31, 34) and in anticipating CIN2 and CIN3 diagnosis (5, 29, 31). However, these trials have also demonstrated a risk of over-

diagnosis and overtreatment if no triage is implemented, particularly in younger women (31, 32). Consequently, the need for specific tests will be even more relevant when the HPV DNA test will be implemented as a primary screening test (12) and in the vaccinated population (19). The most promising target for such novel biomarkers is the E6/E7 viral oncogene expression and its molecular consequences. In fact, once a HPV infection is established in the cervical epithelium, altered transcriptional regulation of the E6/E7 viral oncogenes, which affects almost all of the cellular pathways, is likely to provide the following important step toward malignancy (18, 42). HPV oncogene active transcription and its effects on the host cell can be monitored directly through the detection of E6/E7 viral mRNA transcripts or proteins (9, 25, 35), or indirectly, for example, through the detection of the host protein p16 (3, 6). In fact, it has been widely reported that the p16 expression is affected by the HR-HPV E7 protein and its upregulation in uterine cervix significantly correlates with the increasing severity of the lesions (3, 24, 40).

\* Corresponding author. Mailing address: Agency for Public Health Lazio Sanità, Via di Santa Costanza 53, 00198 Rome, Italy. Phone: 390683060438. Fax: 390683060463. E-mail: giorgirossi@asplazio.it.

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Commercially available robust assays for HPV mRNA detection can be performed in reflex after liquid based cytology or the HPV DNA test (4, 9, 22, 26, 28, 30, 33, 38). We compared here the clinical performance of the PreTect HPV-Proofer E6/E7 mRNA assay (Norchip, Klokkestua, Norway) as a triage test to that of the HPV DNA test and cytology for detecting high-grade cervical intraepithelial neoplasia or worse (CIN2+). The main difference between the HPV mRNA and DNA test is that the former is a type-specific test which detects E6/E7 mRNA of five high-risk oncogenic HPV types (HPV-16, -18, -31, -33, and -45), whereas the latter targets DNA of 13 high-risk oncogenic HPV types (HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, and -68).

We also measured the clinical utility of mRNA in different screening strategies. The rationale to add a triage test in a screening algorithm can be to increase specificity and, at the same time, to increase efficiency, i.e., reducing the number of more expensive and invasive tests. The endpoints used to evaluate clinical utility of the tests under study were the reduction of colposcopies and positive predictive value and sensitivity in identifying CIN2+ lesions.

#### MATERIALS AND METHODS

**Setting.** A retrospective study was performed collecting data from four Italian gynecological prevention clinics—a research hospital, the Regina Elena Cancer Institute of Rome; two medical school hospitals, G. D'Annunzio University of Chieti; and the S. Andrea Hospital, which belongs to the University La Sapienza of Rome—and from one cervical cancer screening center, the F. Renzetti Hospital of Lanciano Vasto. All of the centers are public institution, and virtually all of the tests are paid by the national healthcare system. The Laziosanità Agency coordinated the study and performed the data analyses.

**Patients.** We retrospectively analyzed the clinical history of 1,610 women who underwent an E6/E7 mRNA test on cervical samples collected from January 2004 to December 2006. According to protocols, the women were referred to mRNA only after having undertaken one of the first level tests, i.e., an HPV DNA and/or a cytology positive test.

The inclusion criteria were an mRNA test and an age >18 years. Exclusion criteria were treatment for cervical lesions in the previous 5 years or a history of any prior type of cancer. For all women undergoing the mRNA test, a reference Pap test or HPV test were searched, i.e., the previous test that referred the woman to the mRNA test. This test should have been done within 3 months before the mRNA test. A total of 1,414 patients had an evaluable, conventional or liquid-based, Pap test, and 1,390 patients had an HPV DNA test performed by HC2, PCR, or both tests. In the case of discordant results between the two HPV DNA tests, the positive test was always considered. A total of 1,265 of the 1,610 patients had undergone all three of the tests, i.e., the HPV DNA and mRNA tests and the Pap test. All of the analyses were performed on the subset of women for which the three test results were available. The mean age of the women included was 39.5 years (standard deviation [SD] = 11.3 years, with a range of 18 to 83 years); 95% of them were between 21 and 65 years old.

**Cytological and histological diagnoses.** Cervical samples were taken by using a cytobrush (Cytec, Italy) and plastic Ayre's spatula (Cytec), according to the manufacturer's instructions and stored in 20 ml of PreservCyt solution (Cytec) at 4°C until use. Liquid-based cytology was prepared by using the ThinPrep 2000 System according to the manufacturer's instructions (Cytec). The cytological specimens were reported using the 2001 Bethesda Reporting System. The few ASC-H (atypical squamous cells, cannot exclude H-SIL) results reported were included in the H-SIL (high-grade squamous intraepithelial lesion) group, while the AGC (atypical glandular cells) reports were grouped together with the ASC-US (atypical squamous cells of undetermined significance) group.

Of the 1,610 patients, 843 underwent a colposcopic examination within 2 months from the mRNA test (assessment rate, 52.3%). We assumed that women who had undergone colposcopy without a biopsy had no CIN2 or more serious diagnoses (CIN2-). Cervical biopsy specimens were sampled in 586 patients under a colposcopic guide (biopsy rate, 69.5%). All hematoxylin-eosin slides were diagnosed according to the current World Health Organization classification. The benign cases and mild dysplasia-CIN1 diagnoses are referred to here as

less than CIN2 (CIN2-). Diagnoses of moderate dysplasia-CIN2, severe dysplasia-CIN3-carcinoma *in situ*, and invasive squamous cell carcinoma (SCC) are referred to here as CIN2+. Histological diagnoses were reported as follows: 356 CIN2- (74 tissues within normal limits, along with 282 CIN1) and 230 CIN2+ (120 CIN2, 86 CIN3, and 24 invasive SCC).

**E6/E7 mRNA detection.** Portions (5 ml) of PreservCyt solution were used for the detection of E6/E7 mRNA of HPV types 16, 18, 31, 33, and 45, within 14 days of sample collection, by the PreTect HPV-Proofer Kit (referred to as the mRNA test) (Norchip) according to the manufacturer's instructions. mRNA was extracted by using an RNeasy minikit (Qiagen, Italy). The PreTect HPV-Proofer utilizes an isothermal nucleic acid sequence-based amplification (NASBA) that amplifies mRNA in a DNA background, detecting and genotyping HPV transcripts in the same reaction. The amplified products were detected in real time using fluorescence-labeled molecular beacon probes directed against full-length E6/E7 mRNA. Accumulated mRNA fluorescent profiles were analyzed and assigned a positive or negative status by the supplied PreTect analysis software. Human U1 small ribonucleoprotein (U1A mRNA) was used as an RNA integrity/adequacy internal control. When the U1A amplification was not detected, the test result was deemed invalid.

**HC2 test.** Testing for HR-HPV DNA was principally performed by using the Hybrid Capture 2 (HC2) test (Qiagen), a semiquantitative signal-amplified hybridization assay for the chemiluminescent detection of the 13 most common HR-HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68), as described by the manufacturer. Before performing the HC2 test, 4 ml of PreservCyt solution was processed by using a HC2 sample conversion kit (Qiagen). The positive cutoff (CO) value was considered the mean of the positive control samples. The results were considered positive when the ratio between the relative light units of the sample (RLU) and the chosen positive CO (RLU/CO) was  $\geq 1$ .

**PCR.** For 103 women, HPV infection was determined only by PCR. The PCR HPV test was performed by using an HPV MX BIO kit for HPV DNA amplification, followed by a type-specific reverse dot blot hybridization using the HPV-TYPE kit (Ab Analitica, Italy) (14) for genotyping. The kit amplifies a 449- to 458-bp fragment within the L1 region, together with a 230- to 270-bp fragment within the E6/E7 region of the viral genome. In addition to verify DNA quality, amplification of a 268-bp fragment of the human  $\beta$ -globin gene was performed. This PCR assay is able to recognize 29 high-, intermediate-, and low-risk HPV types (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 61, 66, 68, 70, 72, 73, 81, and 82). Only women who were found to be positive for the same 13 HR-HPV types recognized by the HC2 test were considered HPV DNA positive by PCR.

**Statistical analysis.** The accuracy measures used for detecting CIN2+ in the mRNA test (sensitivity, specificity, positive predictive value [PPV], and negative predictive value [NPV]) and their relative 95% confidence intervals (95% CI) were stratified according to cytological grade and compared to the performance of HPV DNA test (HC2 or PCR test) in the same cytological category, whereas accuracy measures were presented only in the subset of HPV+ women in order to compare it to cytology (at three different thresholds: ASC-US+, L-SIL+, and H-SIL+). The probability of having a colposcopy was strongly influenced by the results of the tests themselves. Consequently, all parameters were adjusted for completeness of the colposcopy follow-up. To explain the algorithm, we took the example of HPV DNA sensitivity in the ASC-US and calculated it according to the following equation:

$$\text{HPV DNA SEN}_{\text{adj}} = \frac{\{[\text{SEN}_{\text{mRNA}+} \cdot (\text{prevCIN2}_{\text{mRNA}+}/\text{complFU}_{\text{mRNA}+})] + [\text{SEN}_{\text{mRNA}-} \cdot (\text{prevCIN2}_{\text{mRNA}-}/\text{complFU}_{\text{mRNA}-})]\}}{[(\text{prevCIN2}_{\text{mRNA}+}/\text{complFU}_{\text{mRNA}+}) + (\text{prevCIN2}_{\text{mRNA}-}/\text{complFU}_{\text{mRNA}-})]}$$

where  $\text{SEN}_{\text{mRNA}+}$  is the sensitivity among mRNA+ samples,  $\text{SEN}_{\text{mRNA}-}$  is the sensitivity among mRNA- samples,  $\text{prevCIN2}_{\text{mRNA}+}$  is the prevalence of CIN2+ among mRNA+ samples,  $\text{prevCIN2}_{\text{mRNA}-}$  is the prevalence of CIN2+ among mRNA- samples,  $\text{complFU}_{\text{mRNA}+}$  is the proportion of women with colposcopic follow-up among mRNA+ samples, and  $\text{complFU}_{\text{mRNA}-}$  is the proportion of women with colposcopic follow-up among mRNA- samples.

The same was determined for the specificity according to the following equation:

$$\text{HPV DNA SPE}_{\text{adj}} = \frac{\{[\text{SPE}_{\text{mRNA}+} \cdot (\text{prevCIN1}_{\text{mRNA}+}/\text{complFU}_{\text{mRNA}+})] + [\text{SPE}_{\text{mRNA}-} \cdot (\text{prevCIN1}_{\text{mRNA}-}/\text{complFU}_{\text{mRNA}-})]\}}{[(\text{prevCIN1}_{\text{mRNA}+}/\text{complFU}_{\text{mRNA}+}) + (\text{prevCIN1}_{\text{mRNA}-}/\text{complFU}_{\text{mRNA}-})]}$$

where  $\text{SPE}_{\text{mRNA}+}$  is the specificity among mRNA+,  $\text{SPE}_{\text{mRNA}-}$  is the sensitivity

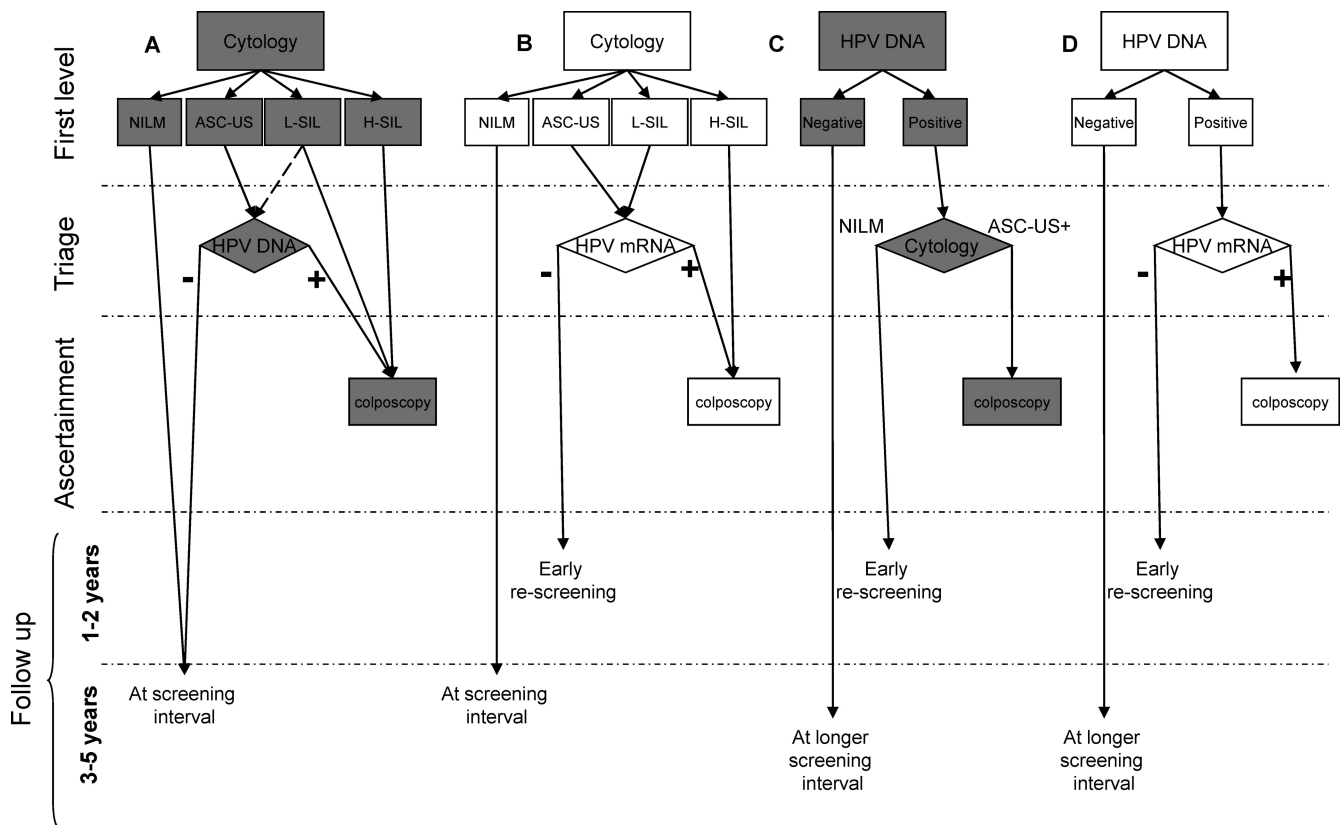


FIG. 1. Screening algorithm used to compare clinical utility of HPV mRNA with that of cytology and HPV DNA testing. NILM, negative for intraepithelial lesions and malignancy; ASC-US, atypical squamous cells of undetermined significance; L-SIL, low-grade squamous intraepithelial lesion; H-SIL, high-grade squamous intraepithelial lesion.

among mRNA- samples,  $\text{prevCIN1-mRNA+}$  is the prevalence of women without CIN2+ among mRNA+ samples,  $\text{prevCIN1-mRNA-}$  is the prevalence of women without CIN2+ among mRNA- samples,  $\text{complFU}_{\text{mRNA+}}$  is the proportion of women with colposcopic follow-up among mRNA+ samples, and  $\text{complFU}_{\text{mRNA-}}$  is the proportion of women with colposcopic follow-up among mRNA- samples.

With regard to mRNA or cytology specificity and sensitivity, all of the adjustments were made using the HPV DNA results. The CI was calculated according to the real number of observed CIN2+ or CIN1-. It is important to note that the adjustments are relevant for specificity, whereas for sensitivity the crude and adjusted estimates are very similar. Table 3 (see below) was the only table not adjusted because it was stratified by both HPV and cytological results.

The proportion of avoided colposcopies, PPV, and sensitivity for CIN2+ detection were reported in order to measure the clinical utility of the mRNA assay as a triage test for ASC-US and/or L-SIL cytology and HPV DNA-positive women. All statistical analyses were performed using STATA software version 8.

## RESULTS

The clinical utility of the Proofer HPV mRNA test was measured under different screening strategies, as shown in Fig. 1. The data stratified by cytological grades of the study population of 1,265 women with a breakdown of HPV DNA and mRNA results along with histological diagnoses are shown in Fig. 2. There were 64 invalid mRNA results overall, the proportion of which dropped in the last year of recruitment. We observed a significant increase in HPV mRNA, as well as DNA presence [both  $p(X^2)$  for trend  $< 0.0001$ ] from the NILM (negative for intraepithelial lesions and malignancy) through to the H-SIL+ specimens. It is worth noting that all of the 64 invalid cases for the mRNA test were HPV DNA positive, but

no histological CIN2+ lesions were found in this group ( $P = 0.012$  compared to mRNA-negative results). Women with invalid results were excluded by the following analyses. The data analyses were conducted on the 1,201 patients who had all three tests valid.

**Accuracy indicators.** The accuracy indicators for mRNA and DNA assays based on 912 women with abnormal cytology are summarized in Table 1. Among all of the cytological grades, the sensitivity for CIN2+ of HPV mRNA was significantly lower than that of HPV DNA. Conversely, mRNA showed a significantly higher specificity compared to HPV DNA. Consequently, the HPV mRNA PPV was always much higher and the NPV was slightly lower than HPV DNA.

Figure 3a shows the sensitivity plotted against 1-specificity for HPV DNA and mRNA tests in women with ASC-US or L-SIL. Given the very low specificity, the area under the curve for HPV DNA was close to 0.5, i.e., no efficiency of the test.

The accuracy indicators for mRNA were determined in comparison with the cytology for 937 HPV DNA-positive women. The results are summarized in Table 2. We compared the performance of HPV mRNA and cytology by considering three different positivity thresholds (ASC-US+, L-SIL+, and H-SIL+). Cytology sensitivity ranged from 92% at the ASC-US+ threshold to 53% at the H-SIL+ threshold. In contrast, cytology specificity was lowest for the ASC-US+ threshold (21%) and highest for H-SIL+ threshold (91%). The HPV mRNA sensitivity and specificity in this set of patients were 68

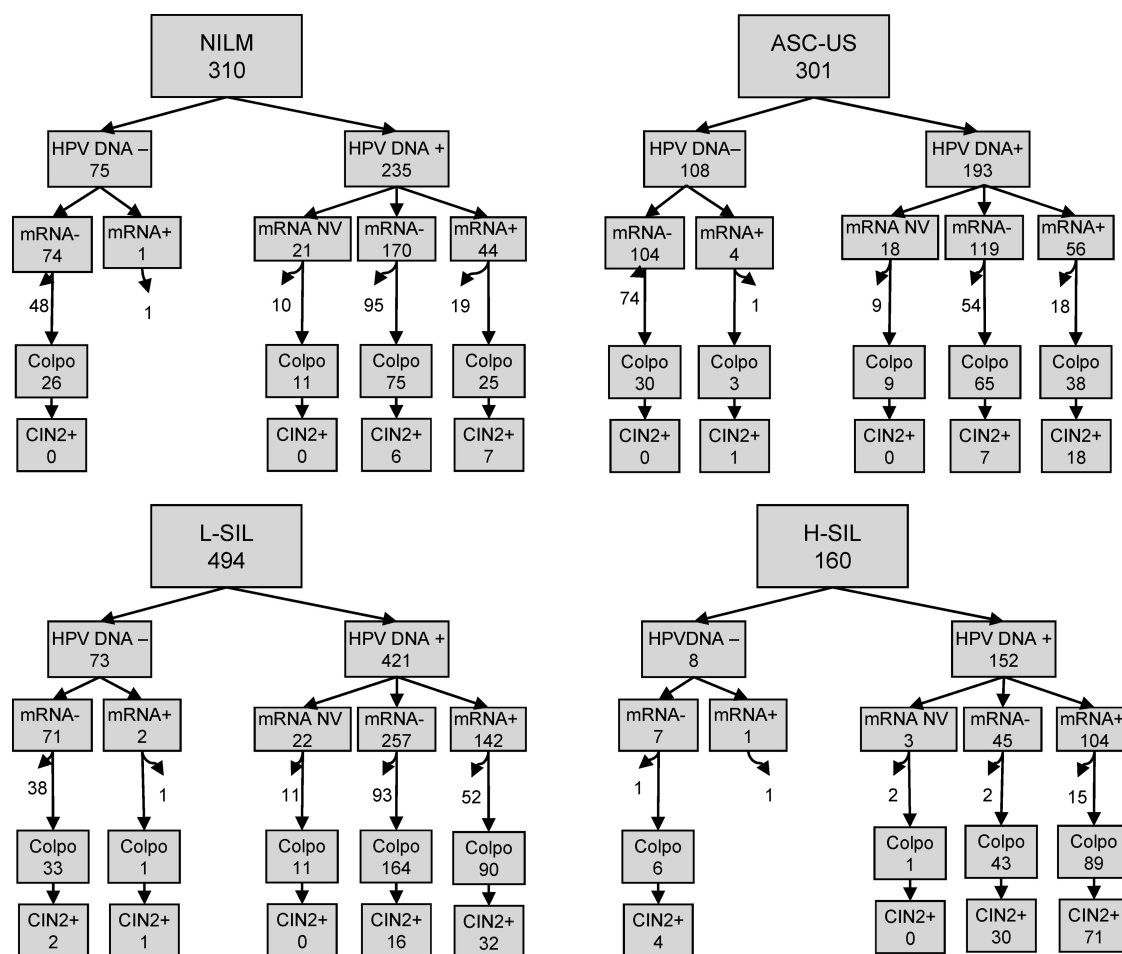


FIG. 2. Summary of results stratified by cytology, HPV DNA and mRNA testing. Cases with no available cytological findings or HPV DNA test results are not reported in the chart. The side arrows report the number of women lost to colposcopy follow-up. NILM, negative for intraepithelial lesions and malignancy; ASC-US, atypical squamous cells of undetermined significance; L-SIL, low-grade squamous intraepithelial lesion; H-SIL, high-grade squamous intraepithelial lesion; colpo, colposcopy; CIN2+, cervical intraepithelial neoplasia grade 2 or more severe diagnosis.

and 72%, respectively. Only 99 HPV DNA-negative women underwent a colposcopy and, among them, we found 8 to be CIN2+. Consequently, the estimates were strongly influenced by chance and are not shown here.

Figure 3b shows sensitivity plotted against 1-specificity for cytology and mRNA testing in HPV DNA-positive women. The mRNA test showed a performance in between the cytology L-SIL+ and H-SIL+ cutoffs.

Among the 187 HC2 HPV DNA-positive CIN2+ cases, 59 were mRNA negative, 24 of which also had a PCR genotyping test. By using PCR, we found that 10 of these 24 patients were infected by HPV types other than 16, 18, 31, 33, and 45. In regard to the two CIN2+ HC2-negative/mRNA-positive cases, the PCR assay confirmed that one was HPV DNA negative, while the other presented only low-risk HPV genotypes.

Accuracy indicators for mRNA in 723 women with abnormal cytology and a positive HPV DNA test are summarized in Table 3. The mRNA PPV was 80% in women with H-SIL+ cytology.

**mRNA assay as a triage test.** As reported in Table 1, the mRNA test was found to be positive in 21% of the ASC-US cases and in 31% of the L-SIL cases, i.e., there were 79 and a 69% reductions in colposcopy referral, respectively. mRNA sensitivity was 83% in ASC-US and 62% in L-SIL cases, i.e., it missed more than one-third of the CIN2+ when the two cytological grades were considered together. In the same population, the HPV DNA test identified more than 90% of the CIN2+ (sensitivities of 99% in ASC-US and 91% in L-SIL), but the reduction in colposcopy referral rate was considerable only for the ASC-US (test positivity rate, 62%; colposcopy reduction, 38% [95% CI = 32 to 44%]). In contrast, it was negligible for L-SIL (test positivity rate, 85%; colposcopy reduction, 15% [95% CI = 12 to 19%]).

Using the mRNA test for triaging HPV DNA-positive women (Table 2), the test positivity rate was 37%, i.e., a colposcopy reduction of 63% (95% CI = 60 to 66%), with a sensitivity of 68% (95% CI = 61 to 75%), losing 32% (95% CI 28 to 43%) of the CIN2+. A cytological triage that considered the ASC-US as the cutoff, would reduce the

TABLE 1. Accuracy indicators for HPV mRNA and DNA assays in 912 women with abnormal cytology shown by cytological grade<sup>a</sup>

Cytology	No. of women tested	No. of women with colposcopy follow-up	No. of CIN2+ women	Test	Test positivity <sup>b</sup>		Sensitivity <sup>c</sup>		Specificity <sup>d</sup>		PPV		NPV	
					%	95% CI	%	95% CI	%	95% CI	%	95% CI	%	95% CI
ASC-US	238	136	26	HPV mRNA	21	17–26	83	63–94	82	73–89	46	31–63	94	87–98
				HPV DNA	62	56–68	99	83–100	29	21–39	22	15–32	99	87–100
L-SIL	472	289	51	HPV mRNA	31	26–35	62	47–75	76	70–81	36	26–47	91	86–94
				HPV DNA	85	81–88	91	80–97	13	9–18	19	14–24	93	80–99
ASC-US and L-SIL	755	425	77	HPV mRNA	27	24–30	67	52–75	45	73–82	80	31–48	31	88–95
				HPV DNA	76	73–79	93	85–98	18	14–22	20	16–25	97	90–100
H-SIL+	157	138	105	HPV mRNA	67	59–74	67	57–76	45	45–84	80	70–88	31	18–45
				HPV DNA	95	90–98	96	91–99	4	0–21	77	68–83	33	4–78

<sup>a</sup> 95% CI, 95% confidence interval; ASC-US, atypical squamous cells of undetermined significance; L-SIL, low-grade squamous intraepithelial lesion; H-SIL, high-grade squamous intraepithelial lesion; CIN2+, cervical intraepithelial neoplasia grade 2 or more severe diagnosis; PPV, positive predictive value; NPV, negative predictive value.

<sup>b</sup> Test positivity and its 95% CI are calculated based on the total number of women tested.

<sup>c</sup> Sensitivity and its 95% CI are calculated based on the number of CIN2+ women, adjusted by follow-up completeness.

<sup>d</sup> Specificity and its 95% CI are calculated based on the number of CIN2– women, i.e., (the number of women with colposcopy follow-up) – (the number of CIN2+ women), adjusted by follow-up completeness.

amount of colposcopies by 23% (95% CI = 20 to 26%) losing 8% (95% CI = 5 to 15%) of CIN2+.

## DISCUSSION

**mRNA accuracy.** The data presented showed that the mRNA positivity rate increased in more severe cytological grades, thus confirming the results of other studies (2, 4, 26, 33, 37, 39). In addition, as described by other authors (26, 38, 39), considering histologically confirmed CIN2+ as the endpoint, the mRNA assay appeared to be more specific but less sensitive than the HPV DNA test. Our data show a strong inverse relation between the specificity of the molecular tests, both HPV mRNA and DNA, and the prevalence of test positivity. As a result, the specificity was higher in ASC-US than in H-SIL, both for HPV DNA and mRNA. This phenomenon has been reported by other studies (20, 22, 30, 38). This association makes it not reasonable to present estimates for the overall specificity. On the contrary, the sensitivity is more stable: 67% in L-SIL/ASC-US and in H-SIL.

On average, the mRNA performance was comparable to cytological examination when plotted in the sensitivity 1–specificity plan but much better than that of the HPV DNA test. This is probably due to the very high prevalence of HPV DNA positivity in our population that is referred to undergo mRNA testing.

Even if the study was not designed to investigate the HPV type-specific analytical accuracy of the test and only few cases were typed, it is worth noting that only the minority of the CIN2+ undetected by the mRNA was due to those same HPV types not being included in the Proofer.

In our series, the proportion of the 264 HPV DNA-negative women that were found to be mRNA positive was very low (8/264 [3%]) but different from zero. These data agree with those of other studies (22, 39) that also found mRNA positivity in a small percentage of the HR-HPV negative samples, both CIN2+ or CIN2–, using the HC2 or the Amplicor test to detect HR-HPV.

Finally, in regard to the invalid results of the mRNA test, we

want to point out that, although all of the 64 mRNA invalid cases were HPV DNA positive, we did not find any CIN2+ lesions among them. This finding strongly suggests that the internal control for the presence of human mRNA was not correctly set up by the producer and was probably corrected in the more recently released lots.

**mRNA as triage test.** The aims of the triage should be to reduce the amount of colposcopies performed and to increase the PPV of colposcopy referral (see Fig. 1). These advantages often have the unavoidable cost of decreasing sensitivity. Consistently with previous results (22, 30, 38), as a triage test of cytology (ASC-US and L-SIL) (see Fig. 1B), the mRNA test showed a strong reduction in colposcopies. On the other hand, the sensitivity was low and comparable to that of repeating the cytological test (7, 41), one of the most commonly used strategies used to avoid direct colposcopy referral for women with L-SIL. In contrast, the triage with HPV DNA (see Fig. 1A) showed a relevant colposcopy reduction only in the ASC-US category and not in the L-SIL group. These results are in line with the recent meta-analysis by Arbyn et al. (1). The large proportion of missed CIN2+ does not allow the referral of mRNA negative women at a normal screening interval, as it is now feasible if the HPV DNA test is used for triaging women with ASC-US cytology. Furthermore, given the reduced number of HR-HPV types included in the PreTect HPV-Proofer test, we suppose that for some mRNA-negative women with persistently positive cytology, a colposcopy would be recommended at some time, resulting in a partially contradictory algorithm.

Recently, Sorbye et al. (36) observed a 96% mRNA PPV in women with H-SIL cytology. Consequently, these authors proposed a “test and treat” protocol in order to avoid the colposcopy-guided biopsy step for these women. In our sample, the mRNA PPV in women with H-SIL cytology was only 80%. The difference may be partially due to local differences in cytology specificity or in colposcopy sensitivity (i.e., a lower biopsy rate in our study). In any case, a “test and treat” strategy cannot be universally recommended and re-

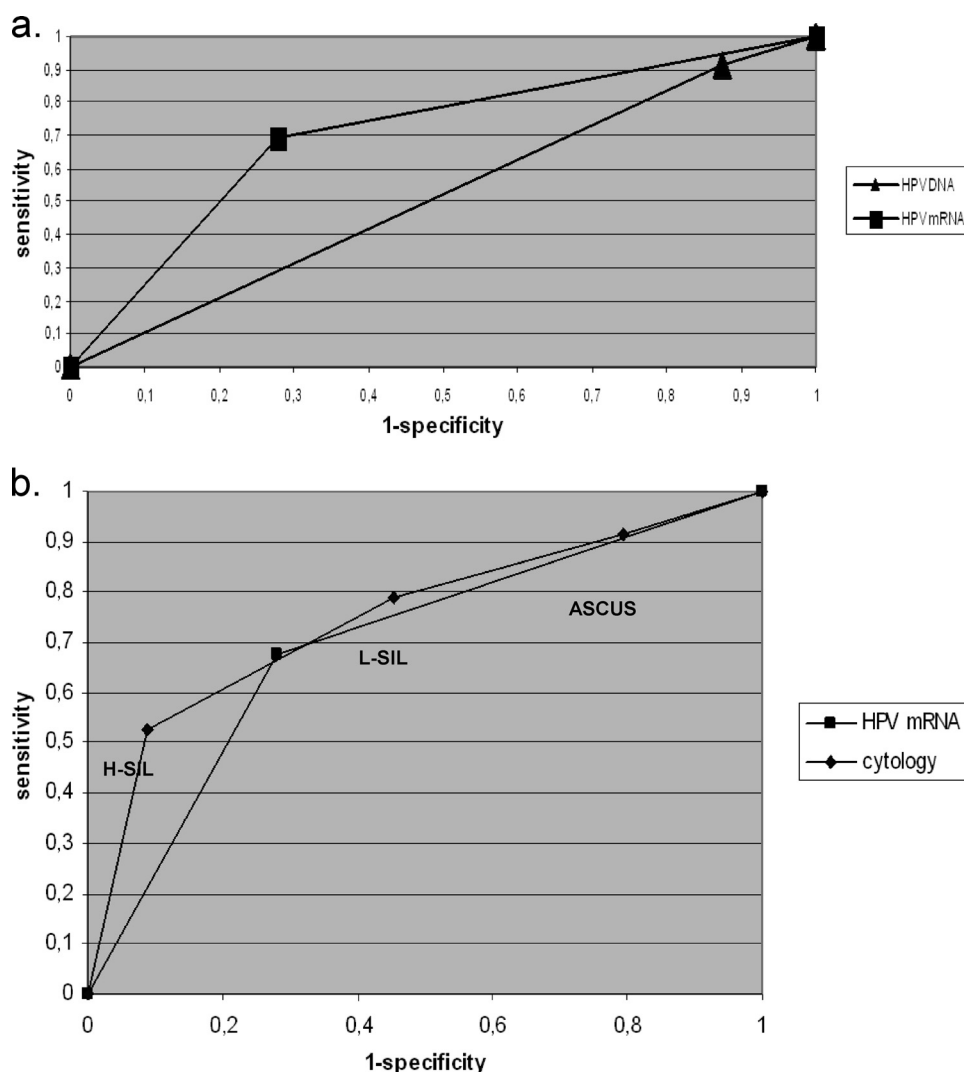


FIG. 3. (a and b) ROC curves for HPV DNA and HPV mRNA tests in women with ASC-US or L-SIL cytology (a) and for HPV mRNA test and cytology at the ASC-US+, L-SIL+, and H-SIL+ cutoffs in HPV DNA-positive women (b). ASC-US, atypical squamous cells of undetermined significance; L-SIL, low-grade squamous intraepithelial lesion; H-SIL, high-grade squamous intraepithelial lesion.

quires accurate local measurement of the actual PPV before implementation.

In HPV DNA-positive women, mRNA test as a triage test (see Fig. 1D) reduced the colposcopic referral more drastically compared to cytology (see Fig. 1C), but at the cost of a lower sensitivity. It must be noted that cytology sensitivity is greatly

overestimated in our population, since the proportion of cytology-negative cases among the HPV-positive women was much smaller than in the screening population, as explained below in the limitation section. Nevertheless, given the high number of CIN2+ found in the HPV DNA-positive/mRNA-negative women, this group should undergo a strict follow-up,

TABLE 2. Accuracy indicators for HPV mRNA and cytology in 937 HPV DNA-positive women<sup>a</sup>

Test	Test positivity <sup>b</sup>		Sensitivity <sup>c</sup>		Specificity <sup>d</sup>		PPV		NPV	
	%	95% CI	%	95% CI	%	95% CI	%	95% CI	%	95% CI
HPV mRNA	37	34–40	68	61–75	72	66–75	53	47–60	84	80–88
Cytology ASC-US+	77	74–80	92	87–95	21	16–24	35	31–40	88	80–94
Cytology L-SIL+	58	55–61	79	73–85	55	48–58	44	38–50	85	80–89
Cytology H-SIL+	16	14–18	53	46–60	91	88–94	77	68–83	82	78–85

<sup>a</sup> Abbreviations are as defined in Table 1, footnote a.

<sup>b</sup> Test positivity and its 95% CI are calculated based on the 937 tested women.

<sup>c</sup> Sensitivity and its 95% CI are calculated based on the 187 CIN2+ women found, adjusted by follow-up completeness.

<sup>d</sup> Specificity and its 95% CI are calculated based on the 254 CIN2– women found, adjusted by follow-up completeness.

TABLE 3. Accuracy indicators of HPV mRNA test in 723 women with abnormal cytology and HPV DNA positive test shown by cytological grade<sup>a</sup>

Cytology	No. of women tested	No. of women with colposcopy follow-up	No. of CIN2+ women	Test positivity <sup>b</sup>		Sensitivity <sup>c</sup>		Specificity <sup>d</sup>		PPV		NPV	
				%	95% CI	%	95% CI	%	95% CI	%	95% CI	%	95% CI
ASC-US	175	103	25	47	38–56	72	51–88	74	63–84	47	31–64	89	79–96
L-SIL	399	254	48	36	31–41	67	52–80	72	65–78	36	26–46	90	85–94
H-SIL+	149	132	101	70	62–77	70	60–79	42	25–61	80	70–88	30	17–46

<sup>a</sup> Abbreviations are as defined in Table 1, footnote *a*.

<sup>b</sup> Test positivity and its 95% CI are calculated based on the total number of tested women.

<sup>c</sup> Sensitivity and its 95% CI are calculated based on the number of CIN2+ women.

<sup>d</sup> Specificity and its 95% CI are calculated based on the number of CIN2– women, i.e., (the number of women with colposcopy follow-up) – (the number of CIN2+ women).

at least once a year. The discrepancy between the HPV types included in HPV DNA tests and the mRNA PreTect HPV-Proofer will compel practitioners to refer the mRNA-negative women with persistent HPV DNA infection to undergo a colposcopy. In fact, HPV types other than the five included in the PreTect HPV-Proofer may account for up to 7% (15) of invasive cancers in Europe, even if these types are much slower in neoplastic transformation (23). The APTIMA test, able to detect E6/E7 mRNA from 14 HR-HPV types without typing, has also been evaluated as a triage marker for abnormal cytology (17), and it has been compared to the PreTect HPV-Proofer assay (38). The APTIMA test showed a very high clinical sensitivity for both CIN2+ and CIN3+ endpoints (8, 17), possibly due to the broad spectrum of HPV types that it recognizes. However, adding carcinogenic HPV types in the mRNA tests may negatively influence the test specificity. In fact, the PreTect HPV Proofer test in the Szarewski study (38) showed a significantly higher specificity compared to the APTIMA test, despite the lower sensitivity.

Recently, p16 overexpression has been proposed as a HPV triage test (6). It has been demonstrated that it was able to decrease the colposcopic rate as cytology, showing, at the same time, a higher sensitivity than cytology, only slightly lower than that of a direct referral to colposcopy of all HPV DNA-positive women. However, in the under-35-year age group, the detection rate was about three times that found by a cytology based protocol, likely representing an overdiagnosis leading to relevant overtreatment of regressive high-grade lesions. In fact, it is known that most CIN2, and also some CIN3, lesions spontaneously regress, particularly in younger women (27). Overdiagnosis and overtreatment of regressive high-grade lesions is the main limitation to the application of HPV DNA test in younger women. For these women, it could be speculated that a cross-sectional low sensitivity of a triage test is not a limit in itself, rather a necessary, although not sufficient, requirement to be able to recognize preinvasive lesions with different progression risks. Moreover, according to the current knowledge about the predominant role of HPV16 in early-onset cervical cancers (15), it may be expected that the reduced number of types in the PreTect HPV-Proofer mRNA test reduces the treatment of regressive high-grade lesions without missing the relevant ones, at least in younger women. Only a prospective study evaluating the prognostic role of the mRNA test including only five high-risk types in CIN patients may clarify

whether lower sensitivity corresponds to a greater ability in distinguishing lesions with different probability to persist and progress, or if it is simply an analytical limit of the test.

Finally, the PreTect HPV-Proofer mRNA test, like other new HPV tests, gives a type-specific positivity answer. Further investigations are needed to understand the clinical utility of distinguishing HPV types in a screening setting and how to manage a differential follow-up based on type-specific risk of progression.

**Limitations.** Our study population was referred to an mRNA test for several reasons (i.e., an abnormal Pap test or a positive HPV DNA test). Consequently, it differs from a screening population referred to triage testing. Nevertheless, we tried to reduce the effect of the selection bias by calculating the accuracy indicators only for women with a positive cytology or HPV test. In fact, the selection bias is stronger for cytology-negative and HPV-negative women, as shown by the extremely high proportion of HPV DNA-positive results in cytology-negative women and the high proportion of cytology-positive results among HPV-negative women. This bias strongly affects the indicators used to measure clinical utility as a triage test of HPV DNA-positive women: in particular, we underestimated the colposcopy reduction of mRNA and cytology, and we strongly overestimated the sensitivity of cytology.

Moreover, our endpoint, i.e., histologically confirmed CIN2+ lesions, was not blindly reviewed. This represents a limit to estimate the relative sensitivity and specificity of tests in a double testing study (STARD, <http://www.stard-statement.org/>).

Finally, in our database, the percentage of performed colposcopies and, consequently, the ascertainment of the histological endpoint, was on average quite low, and the probability of having a colposcopy was strongly influenced by the results of the test itself. We tried to take into account this bias, adjusting for the completeness of the colposcopic follow-up according to test results.

**Conclusions.** The E6/E7 HPV mRNA assay shows higher specificity but lower sensitivity than the HPV DNA test for the detection of CIN2+. When used as a triage test, the assay drastically reduces colposcopy referral in both ASC-US and L-SIL cases, while HPV DNA can be used only to triage ASC-US. In addition, the mRNA test, when applied as triage of HPV DNA-positive women, reduces the colposcopy referral

more than cytology. However, the low sensitivity for CIN2+ lesions strictly requires a close follow-up for HPV DNA-positive/mRNA-negative women.

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